Downregulation of transient receptor potential M6 channels as a cause of hypermagnesiuric hypomagnesemia in obese type 2 diabetic rats

Kaori Takayanagi,1,2 Taisuke Shimizu,2 Yosuke Tayama,2 Akira Ikari,3 Naohiko Anzai,4 Takatsugu Iwashita,2 Juko Asakura,2 Keitaro Hayashi,4 Tetsuya Mitarai,2 and Hajime Hasegawa2

1Ishikawa Kinenkai Kawagoe Ekimae Clinic, Kawagoe, Saitama, Japan; 2Department of Nephrology and Hypertension, Saitama Medical Center, Saitama Medical University, Kawagoe, Saitama, Japan; 3Laboratory of Biochemistry, Department of Biopharmaceutical Sciences, Gifu Pharmaceutical University, Gifu, Japan; and 4Department of Pharmacology and Toxicology, Dokkyo Medical University School of Medicine, Tochigi, Japan

Submitted 11 November 2013; accepted in final form 11 December 2014

The incidence of hypomagnesemia among patients with diabetes mellitus is ~13.5–47.7%, much higher than the incidence in nondiabetic individuals: 2.5–15% (40, 45, 59). Individuals with metabolic syndrome are also known to demonstrate a high incidence of hypomagnesemia (34). The hypomagnesemia in diabetic patients is thought to participate in the development of hypertension and/or coronary disease as diabetic complications (8, 28). Diminished insulin sensitivity is a principal characteristic feature of obese patients with type 2 diabetes, and the finding that the presence of hypomagnesemia attenuates insulin’s effects on adipose and muscle cells may indicate that hypomagnesemia is also closely involved in the development of insulin resistance (9, 25).

In addition, diminished insulin sensitivity is not appropriately compensated for by β-cell function in individuals with hypomagnesemia, indicating that it could be linked to inadequate β-cell compensation (52). Moreover, the presence of hypomagnesemia in healthy subjects attenuates insulin action (48), and the administration of Mg2+ to those subjects ameliorates the insulin resistance (19). These clinical observations are supported by the finding of a significant inverse relationship between dietary Mg2+ intake and the risk of developing diabetes (54) and the finding that a Mg2+-rich diet reduces the oxidative risk of developing diabetes by 34% in women and 33% in men (37). Therefore, the elucidation of the molecular mechanisms underlying hypomagnesemia may contribute to the development of new clinical approaches to combat diabetes.

Among the suspected causes of hypomagnesemia, reduced Mg2+ intake and loss of Mg2+ from the gastrointestinal tract concomitant with diarrhea has been considered (45). However, the presence of hypermagnesemia in healthy subjects suggests that Mg2+ is absorbed daily from food and drinking water and 8–10% of filtered Mg2+ is absorbed in tubules from 84 mmol/day. In the presence of hypermagnesemia hypomagnesemia suggests that urinary loss resulting from reduced reabsorption in the kidneys is a major cause of hypomagnesemia in diabetes (45). Hypermagnesemia is known to be evident even in the early phase of diabetic nephropathy, showing microalbuminuria without apparent proteinuria and a reduction of glomerular filtration (2, 12). However, it has yet been determined whether hypermagnesemia is a characteristic pathological feature of diabetic nephropathy or an indirect phenomenon associated with other renal disorders, such as interstitial impairment.

To maintain Mg2+ balance in the human body, ~12 mmol Mg2+ is absorbed daily from food and drinking water and 8 mmol is excreted in feces and 4 mmol in urine (56). The kidneys have a central role in Mg2+ metabolism because each day, 80 mmol Mg2+ is reabsorbed in tubules from 84 mmol filtered by the glomeruli (17). The major tubular subsegments for Mg2+ reabsorption are the proximal tubules (PTs), thick ascending limb of Henle (TAL), and distal convoluted tubules (DCTs), which are responsible for reabsorbing 10–30%, 40–70%, and 5–10% of filtered Mg2+, respectively (17). In the...
TAL, Mg$^{2+}$ is reabsorbed through a paracellular pathway formed by claudin-16 (paracellin-1) and claudin-19 in tight junctions (21). Active transcellular salt reabsorption in this segment results in a lumen-positive transepithelial voltage driving the passive paracellular reabsorption of divalent cations such as Mg$^{2+}$ (1, 20). It has been shown that genetic mutations of claudin-16 and claudin-19 cause an inherited renal disorder, familial hypermagnesiuria, hypomagnesemia with hypercalciuria and nephrocalcinosis, indicating that these molecules are critical for Mg$^{2+}$ reabsorption (30, 53). In contrast, in DCTs, the transient receptor potential (TRP)M6 channel provides a transcellular pathway for Mg$^{2+}$ reabsorption (1, 31), which is responsible for the final regulation of urine Mg$^{2+}$ output. Although the amount of reabsorbed Mg$^{2+}$ in this segment is not abundant, the DCT is a critical segment because TRPm6 mutation causes a genetic disorder with hypermagnesiuric hypomagnesemia (51).

It is well established that the severity of tubulointerstitial nephropathy (TIN) is a major factor affecting the loss of kidney function (42, 49), and TIN has a greater effect than glomerular nephropathy (TIN) is a major factor affecting the loss of kidney function (42, 49), and TIN has a greater effect than glomerular nephropathy (16). In diabetic nephropathy, TIN is also known to be concomitantly injury on the progression to end-stage renal damage (16). A new therapeutic strategy for the clinical management of diabetic nephropathy is needed. Present findings may indicate a possible role of the detection of molecule resulting in the hypermagnesiuria in diabetes is TRPm6 in DCTs and that the development of hypermagnesiuria and that of TIN were dissociated. We believe that the present findings may indicate a possible role of the detection of hypermagnesiuria as a new parameter to assess the functional alteration of the kidneys in early diabetes and may also provide a new management strategy for the clinical management of diabetes mellitus.

**MATERIALS AND METHODS**

*Animals and experimental design.* Six-week-old male Otsuka Long-Evans Tokushima fatty (OLETF) rats and Long-Evans Tokushima Otsuka (LETO) rats, controls for OLETF rats, were obtained from the Otsuka Pharmaceutical Tokushima Research Institute (Tokushima, Japan). The animal protocol was approved by the Animal Care and Use Committee of Saitama Medical University. For the entire experimental period, rats were housed individually and given free access to food (rodent standard pellet chow, CE-2, CLEA Japan, Tokyo, Japan) and water. The rats’ blood pressure was measured by the tail-cuff method (BP98-A, Softron, Tokyo, Japan). After urine collection for 24 h, blood samples were obtained from the aorta, and the rats’ kidneys were perfused with ice-cold PBS (pH 7.4) and quickly removed under anesthesia (50 mg/kg ip pentobarbital) at 16, 24, and 34 wks of age (n = 6). For the biochemical analysis of blood and urine samples, except Mg$^{2+}$, all were measured according to published methods (3, 57). Serum and urine Mg$^{2+}$ concentrations were measured by colorimetric tests using xylene blue. The measurement of all biochemical parameters was consigned to SRL (Tachikawa, Tokyo, Japan).

*Histology and immunohistochemistry.* Kidneys were snap frozen in liquid nitrogen. Frozen sections (5 μm) were incubated with rabbit anti-anti-claudin-16 antibody (1:500, Invitrogen/Life Technologies, Carlsbad, CA) for 16 h at 4°C, Alexa fluor 594 donkey anti-rabbit IgG (H+L, Invitrogen/Life Technologies) was subsequently applied as a secondary antibody to sections for 60 min at room temperature. Subsequently, after a wash, Alexa fluor 594 mouse anti-zonula occludens-1 antibody (1:200, Invitrogen/Life Technologies) as a third antibody was applied to sections for 60 min at room temperature. Other tissue blocks of kidneys were fixed in PBS containing 4% formaldehyde and embedded in paraffin. Sections (4 μm) were prepared and stained with periodic acid-Schiff and Masson trichrome. Immunohistochemical stainings were performed as previously described (3). The other paraffin sections were treated with primary antibody, rabbit anti-TRPm6 (Osemes, Keswick, CA, Australia), diluted 1:15,000, rabbit anti-Na$^{+}$-K$^{+}$ cotransporter (NCC; Merck, Darmstadt, Germany), diluted 1:8,000, mouse anti-α-smooth muscle actin (α-SMA; Progen Biotechnik, Heidelberg, Germany), diluted 1:2,500 for 16 h at 4°C, or mouse anti-Na$^{+}$-Ca$^{2+}$ exchanger 1 (NCX1; Swant Swiss Antibodies, Marly, Switzerland) diluted 1:2,000 for 1 h at room temperature followed by Dako Cytomation Envision Plus System HRP-Labeled Polymer (Dako, Glostrup, Denmark) or Histofine Simple Stain Rat MAX PO (Nichirei Biosciences, Tokyo, Japan). A synthetic peptide from region 1970–2020 of the rat TRPm6 amino acid sequence, corresponding to “CGKRLPDLKRDYSHARHCNLKATTTPEAEHPERDRNPSLEDHTRL...” conjugated to an immunogenic carrier protein, was used as the antigen. To confirm the specificity of anti-TRPm6 antibody to TRPm6 protein, TRPm6 antibody was preabsorbed with control peptide (2.5 μg/ml, Osemes) for 2 h at room temperature. A liquid diaminobenzidine substrate chromogen system was used for visualization with counterstaining by hematoxylin. The interstitial area was analyzed by subtracting the tubules, glomeruli, and blood vessels from the total area and then dividing by the total area as previously reported (62). We also semi-quantitatively assessed the immunoreactivities of TRPm6, NCC, and NCX1 by referring to Ledeganck’s method (32). The diaminobenzidine-stained area was measured as a percentage of each field from the cortex. All analyses were performed using Image J computer program (1.43u, National Institutes of Health).

**Gene expression analysis.** Total RNA was extracted from the whole kidney, and real-time PCR was used for the assessment of gene expression as previously described (57). TaqMan probes and primers for claudin-16 [assay identifier (ID): Rn00590854_m1], TRPM6 (assay ID: Rn01760130_m1), TRPM7 (assay ID: Rn00586779_m1), Na$^{+}$-K$^{+}$-2Cl$^{-}$ cotransporter (NKCC2) (assay ID: Rn00569491_m1), NCC (assay ID: Rn00571074_m1), monocyte chemotactic protein-1 (assay ID: Rn05806555_m1), and α-SMA (assay ID: Rn01759928_g1) were applied to TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA). TaqMan real-time PCR probe and primers for Na$^{+}$/K$^{+}$ exchanger (NHE3) were designed using Primer Express software (Applied Biosystems, NHE3 probe: 5'-TCCACGCACCCGGGCTCTATGAT-3', forward primer: 5'-TCCACCCGAGCAGCAATAGG-3', and reverse primer: 5'-GCACGTCAGATTTTCTCTGACGCTAA-3'). We used the expression of rat actin-β (Rat ACTB Endogenous Control VIC/MGB Probe, Applied Biosystems) as an internal control for correction of sample variability. We evaluated the expression of each gene by the ΔCt method, where Ct is threshold cycle, as previously reported (62). We also semi-quantitatively assessed the immunoreactivities of TRPm6, NCC, and NCX1 by referring to Ledeganck’s method (32). The diaminobenzidine-stained area was measured as a percentage of each field from the cortex. All analyses were performed using Image J computer program (1.43u, National Institutes of Health).

**Statistical analysis.** Results are presented as means ± SE in each group. Student’s two-tailed t-test was used to compare data between OLETF and LETO rats. Corrected kidney weight, serum Mg$^{2+}$, plasma glucose, and insulin at 16, 24, and 34 wk of age were compared with one-way ANOVA followed by Bonferroni’s post hoc test. P values of <0.05 were considered significant. All analyses were performed using SPSS (version 20.0) for Windows XP (IBM, Armonk, NY).
Table 1. Blood biochemical data of LETO and OLETF rats

<table>
<thead>
<tr>
<th></th>
<th>16 Weeks</th>
<th>24 Weeks</th>
<th>34 Weeks</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight, g</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LETO</td>
<td>426.4 ± 13.4</td>
<td>464.0 ± 4.2</td>
<td>508.8 ± 10.5</td>
</tr>
<tr>
<td>OLETF</td>
<td>579.0 ± 12.7†</td>
<td>622.0 ± 11.9†</td>
<td>673.3 ± 11.0†</td>
</tr>
<tr>
<td><strong>Mean blood pressure, mmHg</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LETO</td>
<td>97.3 ± 3.3</td>
<td>96.5 ± 0.5</td>
<td>95.40 ± 0.9</td>
</tr>
<tr>
<td>OLETF</td>
<td>117.8 ± 2.6†</td>
<td>120.2 ± 1.6†</td>
<td>125.0 ± 1.2†</td>
</tr>
<tr>
<td><strong>Kidney weight, mg/g body wt</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>LETO</td>
<td>3.33 ± 0.06</td>
<td>3.04 ± 0.09</td>
<td>2.90 ± 0.07</td>
</tr>
<tr>
<td>OLETF</td>
<td>4.10 ± 0.26</td>
<td>3.50 ± 0.10†</td>
<td>3.22 ± 0.08†</td>
</tr>
</tbody>
</table>

### Blood

<table>
<thead>
<tr>
<th></th>
<th>LETO</th>
<th>OLETF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Creatinine, mg/dl</td>
<td>0.29 ± 0.01</td>
<td>0.39 ± 0.01</td>
</tr>
<tr>
<td>Na⁺, mmol/l</td>
<td>0.22 ± 0.02†</td>
<td>0.26 ± 0.01†</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>147.8 ± 0.86</td>
<td>148.8 ± 0.40</td>
</tr>
<tr>
<td>Insulin, ng/ml</td>
<td>1.38 ± 0.27</td>
<td>1.40 ± 0.20</td>
</tr>
<tr>
<td>Mg²⁺, mg/dl</td>
<td>2.22 ± 0.08</td>
<td>2.32 ± 0.05</td>
</tr>
<tr>
<td>Plasma aldosterone, pg/ml</td>
<td>358.2 ± 35.9</td>
<td>343.1 ± 27.3</td>
</tr>
</tbody>
</table>

### Urine

<table>
<thead>
<tr>
<th></th>
<th>LETO</th>
<th>OLETF</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine volume, ml/day</td>
<td>20.7 ± 3.0</td>
<td>11.1 ± 1.6</td>
</tr>
<tr>
<td>Na⁺, mmol/l</td>
<td>44.8 ± 1.85</td>
<td>73.0 ± 8.14</td>
</tr>
<tr>
<td>Mg²⁺, mg/dl</td>
<td>6.16 ± 0.68</td>
<td>10.2 ± 1.24</td>
</tr>
<tr>
<td>Glucose, mg/dl</td>
<td>12.8 ± 1.7</td>
<td>15.3 ± 1.9</td>
</tr>
<tr>
<td>Urine excretion of glucose, μg·min⁻¹·100 g body wt⁻¹</td>
<td>0.39 ± 0.01</td>
<td>0.23 ± 0.03</td>
</tr>
<tr>
<td>Urine excretion of Na⁺, μg·min⁻¹·100 g body wt⁻¹</td>
<td>0.15 ± 0.02</td>
<td>0.12 ± 0.02</td>
</tr>
<tr>
<td>Urine excretion of Mg²⁺, μg·min⁻¹·100 g body wt⁻¹</td>
<td>0.20 ± 0.02</td>
<td>0.16 ± 0.01</td>
</tr>
</tbody>
</table>

Values are means ± SE. LETO rats, Long-Evans Tokushima Otsuka rats; OLETF rats, Otsuka Long-Evans Tokushima fatty rats. †P < 0.01 vs. LETO rats; *P < 0.05 vs. LETO rats.
RESULTS

The results of the blood and urine tests are shown in Table 1. Body weights of OLETF rats were significantly larger than those of LETO rats at all ages. The mean blood pressure of OLETF rats was also higher than that of LETO rats. Corrected kidney weights of OLETF rats were decreased with week age (i.e., 16, 24, and 34 wk of age), and they were significantly lower than those of LETO rats at 24 and 34 wk old. Serum creatinine levels of OLETF rats were significantly lower than those of LETO rats at all week ages, reflecting the difference of glomerular filtration due to an increase in the extracellular fluid (ECF) volume.

Hyperglycemia and hyperinsulinemia in OLETF rats were significant at all week ages, although differences between each week age were not observed. Urine Mg²⁺ excretion, assessed by the absolute amount of urine Mg²⁺ output (UMgV; in μg·min⁻¹·100 g body wt⁻¹), was significantly increased after 24 wk, and significant hypomagnesemia compared with LETO rats was apparent in 34-wk-old OLETF rats. In accordance, the fractional excretion of Mg²⁺ (FEMg) was also significantly increased at 24 and 34 wk. The urine albumine-to-creatinine ratio was significantly high in OLETF rats at all week ages. Even at 16 wk old, OLETF rats showed significant albuminuria, indicating that the early phase of OLETF in this study might correspond to the microalbuminuric stage of diabetic nephropathy. In addition, urine glucose excretion corrected by body weight (UGluV) was significantly high in LETO rats at 16 wk; however, urine excretion of Mg²⁺ (UMgV and FEMg) and urine excretion of Na⁺ [absolute amount of urine Na⁺ output (UNaV) and fraction excretion of Na⁺ (FENa)] were not different between LETO and OLETF rats. In contrast, UMgV and FEMg were significantly high in OLETF rats, although there was no significant difference in UGluV, UNaV, and FENa between LETO and OLETF rats at both 24 and 34 wk. Serum Mg²⁺ concentrations were not different among all week ages in both LETO and OLETF rats.

As shown in Fig. 1A, expression of claudin-16 mRNA did not differ significantly between LETO and OLETF rats throughout the experimental period, without time-differential changes in mRNA expression in both LETO and OLETF rats (16 wk: 100.1 ± 2.8% in LETO rats and 87.0 ± 4.6% in OLETF rats, 24 wk: 89.5 ± 4.6% in LETO rats and 100.9 ± 3.5% in OLETF rats, and 34 wk: 90.4 ± 4.5% in LETO rats and 100.9 ± 3.6% in OLETF rats, n = 6, respectively). Dual immunostaining with claudin-16 and zonula occludens-1 antibodies was preceded and conducted to confirm whether the used claudin-16 antibody properly recognized claudin-16 in the tight junction (Fig. 1B). As described in a previous report using the same antibody (24), the majority of both signals were...
colocalized in the basolateral side of the TAL. Immunofluorescent staining of claudin-16 also did not show any apparent difference between LETO and OLETF rats throughout the experimental period (Fig. 1C).

We next studied the expression of Mg\(^{2+}\)-transporting molecules, located principally in DCTs. Figure 2 shows significant and stable inhibition of mRNA expression of TRPM6 in OLETF rats (16 wk: 100.1 ± 3.3% in LETO rats and 59.9 ± 1.7% in OLETF rats, 24 wk: 92.0 ± 5.1% in LETO rats and 61.6 ± 1.5% in OLETF rats, and 34 wk: 85.5 ± 5.6% in LETO rats and 63.0 ± 3.5% in OLETF rats, n = 6, respectively), whereas mRNA expression of TRPM7 did not differ during the experimental period (16 wk: 100.2 ± 3.3% in LETO rats and 109.6 ± 4.6% in OLETF rats, 24 wk: 106.2 ± 2.7% in LETO rats and 114.5 ± 4.5% in OLETF rats, and 34 wk: 127.2 ± 3.1% in LETO rats and 130.3 ± 3.3% in OLETF rats, n = 6, respectively). We also studied mRNA expression of molecules that might affect tubular Mg\(^{2+}\) reabsorption, mRNA expression of both NHE3 and NKCC2 showed no significant differences between LETO and OLETF rats in all of the experimental periods (NHE3: 100.5 ± 5.2% in LETO rats and 110.7 ± 7.2% in OLETF rats at 16 wk, 95.5 ± 4.8% in LETO rats and 108.8 ± 4.9% in OLETF rats at 24 wk, and 105.9 ± 4.6% in LETO rats and 116.1 ± 3.2% in OLETF rats at 34 wk, n = 6, respectively; and NKCC2: 100.0 ± 1.8% in LETO rats and 78.8 ± 8.5% in OLETF rats at 16 wk, 78.1 ± 4.4% in LETO rats and 89.8 ± 4.6% in OLETF rats at 24 wk, and 76.7 ± 3.7% in LETO rats and 85.2 ± 2.6% in OLETF rats at 34 wk, n = 6, respectively).

Before assessing the difference in TRPM6 protein expression by immunostaining, we checked the specificity of the anti-TRPM6 antibody. As shown in Fig. 3A, when TRPM6 antibody was preabsorbed with control peptide, no apparent immunoreactivity was detected, indicating that the antibody would specifically recognize TRPM6 protein, as previously demonstrated. The localization of TRPM6 immunoreactivity was confirmed by concomitant immunostaining with NCX1, which is known to be principally expressed on the basolateral plasma membrane of DCTs. The TRPM6 signal was detected in the common tubules, which were stained by NCX1 antibody in serial sections (Fig. 3B). We qualitatively evaluated the protein expression of TRPM6 by immunohistochemistry using low-magnified kidney transverse sections. As shown in Fig. 3C, TRPM6 immunoreactivity in OLETF sections was evidently low compared with LETO sections, a finding that was consistent with the results of our mRNA expression analysis.

Observation under high magnification showed that the positive signals of TRPM6 observed in the cortex of OLETF rats were markedly diminished or nearly disappeared in the LETO cortex (Fig. 4A). Since the thiazide-sensitive NCC is known to be a major regulator of TRPM6 expression (44), we also evaluated its expression profile. As shown in the representative micrographs, the cortical signals of NCC were clearly diminished in OLETF sections (Fig. 4B), a result that is consistent with the significant downregulation of NCC mRNA (Fig. 4C). For the semiquantitative assessment of the immunoreactivities of TRPM6 and NCC, we measured the positive area of these immunoreactivities by computer-assisted imaging analysis on multiple sections using pictures converted to monochrome twogradation (postconversion), as shown in the representative pictures in Fig. 4D, top. As a result, both positive areas were significantly and simultaneously decreased in OLETF rats at all week ages (TRPM6: 0.49 ± 0.04% in LETO rats and 0.10 ± 0.01% in OLETF rats at 16 wk, 0.52 ± 0.03% in LETO rats and 0.10 ± 0.01% in OLETF rats at 24 wk, and 0.48 ± 0.02% in LETO rats and 0.12 ± 0.02% in OLETF rats at 34 wk; NCC: 1.59 ± 0.09% in LETO rats and 0.77 ± 0.05% in OLETF rats at 16 wk, 1.69 ± 0.13% in LETO rats and 0.84 ± 0.05% in OLETF rats at 24 wk, and 1.99 ± 0.13% in LETO rats and 0.80 ± 0.06% in OLETF rats at 34 wk; Fig. 4D, bottom). However, as shown in Fig. 5A, apparent differences in the morphology of DCTs were not observed between LETO and OLETF rats. In addition, protein expression of NCX was semiquantitatively analyzed by measurements of the relative area of NCX immunoreactivity to rule out the possibility that the membrane protein might be generally diminished in DCTs of OLETF rats. As shown in Fig. 5, B and C, no significant difference in NCX expression was detected between LETO and OLETF rats.

Finally, to evaluate the correlation between TRPM6 expression and TIN, we measured the relative change in the interstitial area. As shown in representative micrographs (Fig. 6A), expansion of the interstitial area in OLETF rats was not evident compared with that in LETO rats at all week ages, and the statistical analysis among multiple measurements of the relative interstitial area confirmed that there were no significant differences between LETO and OLETF data (Fig. 6B). We also studied the mRNA expression of proinflammatory cytokines related to interstitial fibrosis for the assessment of the development of TIN (Fig. 6C). mRNA expression of both monocyte chemotactic protein-1 and α-SMA did not differ significantly between LETO and OLETF rats throughout the experimental period (Fig. 6C). Finally, we confirmed the difference of

---

**Fig. 2.** Difference in gene expressions of transient receptor potential (TRP)M6 and TRP7. Gene expressions of TRPM6, TRPM7, Na\(^{+}\)/H\(^{+}\) exchanger (NHE3), and Na\(^{+}\)-K\(^{+}\)-2Cl\(^{-}\) cotransporter (NKCC2) by RT-PCR are shown. Open bars indicate LETO rats; solid bars indicate OLETF rats. Data are mean ± SE; n = 6. **P < 0.01.
protein expression of α-SMA by immunostaining. As shown in Fig. 6D, there was no apparent difference of α-SMA expression between LETO and OLETF rats.

DISCUSSION

OLETF rats are generally known to show hyperinsulinemia and microalbuminuria at ~18 wk of age, apparent proteinuria at 25–30 wk (10), and histological changes in the glomeruli, including membrane thickening or mesangial matrix expansion, after 40 wk of age (26). Although the week ages of OLETF rats cannot be simply compared with the clinical stages of human diabetic nephropathy, the 16-, 24-, and 34-wk-old OLETF rats used in this study would roughly correspond to the early phase with microalbuminuria, the midphase with apparent proteinuria, and the preadvanced phase without histological alterations, respectively. Hence, time-differential changes in diabetic nephropathy at various stages could be roughly assessed using this animal model.

To avoid the influence of endogenous creatinine clearance being less reliable in rodents for the present evaluation of glomerular filtration, we assessed the rats’ urine Mg\(^{2+}\) excretion by the absolute amount of urine Mg\(^{2+}\) output (UMgV) following Hou et al.’s method (22). According to Hou et al., UMgV is shown to be altered with a positive correlation to FE\(_{\text{Mg}}\), indicating the validity of this parameter for the assessment of urine Mg\(^{2+}\) excretion (22). In fact, calculated FE\(_{\text{Mg}}\) using inulin clearance showed similar differences between LETO and OLETF rats to UMgV (22). In the present study, a significant increase in urine Mg\(^{2+}\) excretion was present before the appearance of apparent proteinuria, and significant hypomagnesemia was evident in OLETF rats at 34 wk with the increase in urine Mg\(^{2+}\) excretion. However, there were no significant differences in serum Mg\(^{2+}\) concentrations among all week age groups in LETO or OLETF rats.

These observations might indicate that OLETF rats well represented the generally observed hypermagnesiuria in early diabetic nephropathy and resultant hypomagnesemia in humans. It is generally believed that the urine volume or ECF volume significantly affects renal Mg\(^{2+}\) excretion. The results of the present study showed that the urine volume in OLETF rats was significantly increased only among 34-wk-old rats, although a significant increase in UMgV was demonstrated in both 24- and 34-wk-old rats. Likewise, a significantly low plasma aldosterone concentration, suggesting increased ECF volume, was observed only in 34-wk-old OLETF rats. These discrepancies do not straightforwardly deny the possible involvement of urine volume or ECF volume in renal Mg\(^{2+}\) excretion, but they strongly suggest that one or more factors that are independent of the effect of urine or ECF volume, or factors that enhance these effects, are involved in the development of hypermagnesuria in diabetic nephropathy. In addition, urine Mg\(^{2+}\) excretion in OLETF rats was higher than that in LETO rats. However, urine Na\(^{+}\) and glucose excretion were not significantly different between the two groups, which strongly suggests that urine Mg\(^{2+}\) excretion would be regu-
lated independently of Na\(^+\) or glucose excretion in this diabetic model.

The contribution of DCTs to the amount of reabsorbed Mg\(^{2+}\) is lesser than that of the TAL. However, it has been elucidated that a loss of function mutation of TRPM6 in patients with primary hypomagnesemia with secondary hypocalcemia results in renal Mg\(^{2+}\) wasting (50) or an inappropriate elevation of the fractional Mg\(^{2+}\) excretion rate with respect to their low serum Mg\(^{2+}\) levels (51), indicating that the diminished Mg\(^{2+}\) reabsorption in DCTs sufficiently evokes hypermagnesiuria by decreased reabsorption through TRPM6.

Although the regulatory mechanisms of TRPM6 expression have not been fully elucidated, interactions between altered TRPM6 expression and various disease settings or drug effects have been identified.

For instance, EGF functions as a positive regulator of TRPM6 (58), and the anti-EGF receptor antibody cetuximab causes hypomagnesemia by impeding EGF-dependent activation of TRPM6 (60). Similarly, hypomagnesemia concomitant with cyclosporin A administration is now known to be caused principally by cyclosporin-induced downregulation of TRPM6 (23). The upregulation of TRPM6 by a Mg\(^{2+}\)-deficient diet

**Fig. 4.** Protein expressions of TRPM6 and Na\(^+\)-Cl\(^-\) cotransporter (NCC). A and B: representative high-magnification pictures of immunostaining with TRPM6 (A) and NCC (B) antibodies. Original magnification: \(\times100\). C: gene expression of NCC. Data are means ± SE; \(n = 6\). D: for the semiquantitative assessment of immunoreactivities of both TRPM6 and NCC, original pictures (preconversion) were converted to monochrome two-gradation pictures (postconversion), and we measured the positive area (black) and calculated the occupying ratio to the entire field of each picture. We used the average value of measurement of eight randomly selected microscopic fields (magnification: \(\times100\)) in each animal in the statistical analysis of six animals. Open bars indicate LETO rats; solid bars indicate OLETF rats. Data are means ± SE; \(n = 6\). *\(P < 0.05\); **\(P < 0.01\).
and by inhibition of aldosterone-induced downregulation of TRPM6 by Mg\(^{2+}\)/H\(^{100}\) supplementation (55) have also been demonstrated. Taken together, all of the experimental evidence indicates that the downregulation of TRPM6 in the present study would sufficiently cause hypermagnesiuric hypomagnesemia in diabetic nephropathy.

Future studies should investigate the involvement of extracellular glucose and insulin concentration, osmolality, Na\(^{+}\)/H\(^{100}\) concentration, advanced glycation end products, and nitric oxide in the regulation of TRPM6. In general, changes in the expression or activity of Mg\(^{2+}\)/H\(^{100}\)-transporting molecules (claudin-16/19 or TRPM6/M7) are not indispensable for the increase or decrease in Mg\(^{2+}\)/H\(^{100}\) absorption in the TAL and PTs. The lumen-positive charge generated by the functional coupling of Na\(^{+}\)/H\(^{100}\) absorption and K\(^{+}\)/H\(^{100}\) excretion in the TAL is a driving force for paracellular Mg\(^{2+}\)/H\(^{100}\) absorption through claudin-16/19 in tight junctions of the TAL. Diminished NKCC2 expression might cause the reduction of the lumen-positive charge, and the increase in NHE3 expression or activity in PTs might diminish passive Mg\(^{2+}\)/H\(^{100}\) absorption in PTs and might also decrease Na\(^{+}\) delivery into the TAL, with a resultant reduction of Na\(^{+}\) absorption and lumen-positive charge. However, no significant changes of mRNA levels of NKCC2 and NHE3 were observed in this study, suggesting that the changes in urine Mg\(^{2+}\) excretion in OLETF rats would be predominantly provoked by the altered expression of TRPM6 in DCTs.

The chronic administration of thiazide is well known to induce hypomagnesemia concomitant with NCC downregulation (44). Additionally, hypomagnesemia is one of the major clinical features in Gitelman syndrome, a hereditary disorder caused by a loss of function mutation of NCC, which indicates a significant reduction of TRPM6 expression resulting in hypomagnesemia (35, 44). In the present study, gene and protein expressions of NCC were significantly diminished, a result that is consistent with the downregulation of TRPM6 through the entire experimental period, suggesting the possible contribution of NCC downregulation to decreased TRPM6 expression. In addition, Loffing et al. (36) reported the structural damage of DCT cells in NCC-deficient mice and thiazide-treated rats. However, our observations of no significant differences in DCT morphology and NCX protein expression in the present study showed the lesser possibility of the involvement of the structural damage in DCTs of OLETF rats. The presumed interaction between NCC and TRPM6 expression was also indicated by Ledeganck et al., who demonstrated TRPM6 downregulation and a simultaneous reduction of NCC expression as a major cause of hypomagnesemia in cases of cyclosporin administration (32). Moreover, it is generally accepted that the phosphorylation of NCC by members of the WNK family (especially WNK4) via Ste20-related proline-alanine-rich kinase/oxidative stress-responsive kinase-1 plays a central role in the regulation of NCC activity and/or sorting to the membrane surface (14, 29). However, roles have been de-
scribed for vasopressin (15, 41) and aldosterone (39, 43) as regulators of NCC transcription.

In the present study, we did not investigate the regulatory mechanism of NCC downregulation in detail, and we observed no differences in aldosterone levels (Table 1), plasma antidiuretic hormone concentrations, or expression of WNK4 (data not shown) between OLETF and LETO rats. At present, the expression profile of NCC in rodent diabetic models is controversial. Although some studies (5, 27) using obese Zucker rats exhibiting diabetes have described a significant increase in the protein expression of NCC, another study (4) using the same model reported no difference in NCC protein expression. Interestingly, Madala et al. (38) reported that NCC expression was declined by 50% in Zucker fatty rats, whereas renin-aldosterone levels were not significantly different, findings that are consistent with those of the present study. Similarly, Riazi et al. (46) revealed that in Zucker fatty rats, acute salt loading induces a significant reduction of NCC expression, whereas aldosterone does not respond to the salt load.

Although the precise mechanism of NCC downregulation was not demonstrated in the present study, the fact that the expression profile of NCC in diabetes was not consistently established in the multiple studies described here, even though the studies used the same model, may correspond to the clinical variation observed, that is, not all diabetic patients show hypomagnesemia. The expression of NCC is highly regulated by aldosterone, indicating that hypermagnesuria through inhibition of TRPM6-NCC would be influenced by differences in the phenotype of each model animal or experimental conditions in animal experiments and by differences in the fluid volume status or living conditions of patients. The specific conditions resulting in the downregulation of TRPM6 remain to be elucidated.

Since TRPM6 forms a functional ion channel complex with TRPM7 (7), we investigated potential changes in TRPM7 expression, and we did not detect a significant alteration in this study. It appears that the regulatory mechanism and tissue distribution of TRPM6 and TRPM7 may be entirely different. For instance, TRPM6 is selectively expressed in the kidney, lung, cecum, and colon, whereas TRPM7 is located throughout the body (18). Mg$^{2+}$ restriction strongly induces upregulation of TRPM6 in the kidney but does not affect TRPM7 (18). Yogi et al. (61) reported that TRPM7 expression is not affected in the condition of decreased Mg$^{2+}$ concentration in the ECF, whereas this condition induces a downregulation of TRPM6 and enhances urine Mg$^{2+}$ excretion. These results clearly indicate that the expressions of TRPM6 and TRPM7 are independently regulated. It is thus understandable that the expressions of TRPM6 and TRPM7 in the present study showed different profiles in OLETF rats, a result that is consistent with those of several previous studies.

In contrast to our results, Lee et al. (33) reported upregulation of TRPM6 and NCC and no difference in claudin-16 expression in streptozotocin-induced diabetic rats. According to their results, urine Mg$^{2+}$ excretion should be de-
creased, although they also observed an increase in urine Mg\(^{2+}\) excretion with no change in serum Mg\(^{2+}\) concentration. Basically, streptozotocin-induced diabetes is a type 1 diabetic model lacking pancreatic insulin secretion. OLETF is a type 2 diabetic model showing hyperinsulinemia. It is thus difficult to compare the results from these two models, and our results should be assessed independently of the results of Lee et al.

In addition, Nair et al. (41) reported that insulin activates membrane trafficking of TRPM6 to the cell surface and stimulates Mg\(^{2+}\) uptake into cells in vitro. However, an acceleration of Mg\(^{2+}\) transport by insulin was not directly demonstrated in that study, and an action of insulin on Mg\(^{2+}\) excretion from kidneys in animals or humans was not shown. The functional correlation between insulin and hypermagnesuric hypomagnesemia in diabetic patients is still obscure.

We recently studied the molecular mechanisms of hypermagnesuric hypomagnesemia in TIN, which is the other major disease setting showing hypermagnesuric hypomagnesemia. In contrast to the present results, a rat model showing glomerular injury-independent TIN demonstrated significant down-regulation of claudin-16 in parallel with the development of interstitial damage and hypermagnesuric hypomagnesemia (T. Shimizu and H. Hasegawa, unpublished observations). These preliminary data might also indicate that TRPM6-induced hypermagnesuric hypomagnesemia is a characteristic feature of altered tubular function in early diabetic nephropathy. The present results indicate that hypermagnesuria in diabetic nephropathy would be caused principally by the attenuated Mg\(^{2+}\) reabsorption through TRPM6 in DCTs, which is not generally observed in nondiabetic animals. The findings of the present study may provide a scientific background for the usefulness of the assessment of Mg\(^{2+}\) excretion as a clinical parameter of tubular impairment in early diabetic patients in combination with the assessment of microalbuminuria, a parameter of early glomerular injury. The present findings also provide a rationale for the clinical approach of active Mg\(^{2+}\) supplementation to inhibit the development of insulin resistance and/or coronary disease.

In summary, the results of the present study showed significant and TIN-independent downregulation of TRPM6 as a possible cause of hypermagnesuric hypomagnesemia in early diabetic nephropathy, suggesting that the detection of hypermagnesuria may be a beneficial parameter for the assessment of functional changes in renal tubules in early diabetic nephropathy. In addition, therapeutic approaches to the functional alteration in DCTs may be a new strategy for the restoration of insulin resistance.

ACKNOWLEDGMENTS

The authors gratefully acknowledge T. Ozawa for technical assistance.

GRANTS

This work was supported by Saitama Medical Center, Saitama Medical University, Financial Supporting Project for Young Investigators 24-C-1-C10.

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

AUTHOR CONTRIBUTIONS


REFERENCES


41. Tawara Y, Hasegawa H, Takayanagi K, Matsuda A, Shimizu T, Akaura J, Iwashita T, Ogawa T, Katoh H, Mitairai T. Prevention from...


